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Increasing oxime efficacy by blood–brain barrier modulation

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ABSTRACT

One of the shortcomings of current treatment of nerve agent poisoning is that oximes hardly penetrate the blood–brain barrier (BBB), whereas nerve agents easily do. Increasing the concentration of oximes in the brain, would therefore provide an attractive approach to improve medical countermeasures. An explanation for limited penetration might be that oximes are substrates for the active P-glycoprotein (Pgp) efflux transporter located in the BBB.

Using quantitative brain microdialysis in rats, the effect of i.v. injected tariquidar, a non-competitive, specific Pgp-inhibitor, on HI-6 levels in blood and brain was investigated. It appeared that tariquidar enhanced HI-6 levels in the brain approximately 2-fold during the first hour after HI-6 administration, whereas plasma levels did not differ between the treatment groups. A subsequent proof-of-concept study in rats showed that soman-induced seizures and convulsions were prevented almost completely when they were, in addition to HI-6 and atropine, pretreated with tariquidar. Moreover, twice as much AChE activity was present in their brains as compared to control rats.

These results in rats indicate that modulation of the BBB by a drug like tariquidar, which is non-toxic by itself, is of great value in enhancing the efficacy of oximes.

1. Introduction

Although current treatment of nerve agent intoxication can reduce lethality, it does not adequately prevent or terminate seizures and subsequent neuropathology (Shih and McDonough, 1997). This might be explained by the fact that treatment drugs not easily enter the brain. Increasing the brain concentration of drugs currently in use for treatment of nerve agent intoxication is therefore generally considered a valuable approach to improve medical countermeasures, both in the pretreatment and in the post poisoning phase.

Organophosphorus nerve agents are potent inhibitors of acetylcholinesterase (AChE). Immediate treatment of nerve agent poisoning consists of administration of atropine sulphate, a (bis)pyridinium oxime and an anti-convulsant. In particular the benefit of oxime therapy against various nerve agents is subject to debate. Experimental data indicate a limited efficacy of oximes (Worek et al., 2007). An explanation might be that whereas nerve agents easily enter the brain, oximes hardly pass the blood–brain barrier (BBB). This implies insufficient reactivation of inhibited AChE in the central nervous system and ongoing accumulation of the excitatory neurotransmitter acetylcholine (ACh) responsible for seizures, convulsions and subsequent neuropathology. Recently, tertiary oximes appeared to improve survival and prevent seizures at high doses following nerve agent poisoning. This showed to be related to the reactivation of brain cholinesterase (ChE) (Shih et al., 2010; Skovira et al., 2010). However, a drawback of these tertiary oximes is that their broad-spectrum efficacy and potency towards nerve agent inhibited ChE is low, but these studies underscribe the importance of centrally effective oximes.

The problem of limited drug efficacy in the brain in nerve agent poisoning parallels that encountered in research towards refractory epilepsy and (brain) tumors. These fields of research have shown that drug resistance occurs due to the presence of membrane embedded efflux transporter proteins in the BBB, such as P-glycoprotein (Pgp) and breast cancer resistance protein (BCRP). Blocking Pgp resulted in higher levels of anti-epileptics in CNS and more successful management of epileptic insults (Brandt et al., 2006; van Vliet et al., 2006).

The objective of the study presented here was to explore the effects of tariquidar, a well known Pgp and BCRP inhibitor, on the penetration of oximes into the brain to improve therapeutic efficacy in nerve agent poisoning. It was hypothesized that the oxime HI-6

Abbreviations: OP, organophosphate; BBB, blood–brain barrier; Pgp, p-glycoprotein; AChE, acetylcholinesterase; BCRP, breast cancer resistance protein (BCRP); TQD, tariquidar.

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might be a substrate for the Pgp efflux-transporter. Blocking of Pgp pumps by the potent, noncompetitive inhibitor tariquidar, would in that case increase brain HI-6 levels. In clinical trials in humans, tariquidar appeared to be tolerable and Pgp-inhibition was demonstrated for 48 h after single dosing (Fox and Bates, 2007; Stewart et al., 2000).

To this end, the effect of tariquidar pretreatment on brain penetration of HI-6 was assessed using quantitative brain microdialysis in rats. This was followed by investigating the HI-6 efficacy enhancing property of tariquidar on the development of cholinergic signs and seizures in a soman-induced seizure rat model.

2. Methods

2.1. Animals

Male albino Wistar rats (260–340 g), obtained from Harlan, Horst, The Netherlands were used in the current study. Prior to the experiments they were housed with 2–3 animals per cage, and allowed to get accustomed to standard conditions for at least 1 week. Temperature was kept at 19–22 °C, relative humidity was maintained at 55–65% and lights were on from 7 am to 7 pm. Acidified water and standard rodent chow (Teklad Global Diet, Harlan, Horst The Netherlands) were available *ad libitum*. All experiments described received prior approval from the Ethical Committee on Animal Experimentation of TNO.

2.2. Chemicals

HI-6 (1-2-hydroxyiminomethyl-1-pyridino-3-(4-carbamoyl-1-pyridino-2-oxapropane dichloride)), 2-PAM (pralidoxime, 2-pyridine aldoxime methyl chloride) and soman (3,3-dimethylbutan-2-yl methylphosphonofluoridate) were obtained from the stocks of TNO Rijswijk, and were of >98% purity. Tariquidar (XR9576) was kindly provided by Avaant Pharmaceuticals Pvt. Ltd. All other chemicals were purchased from renowned companies and of standard purity.

2.3. In vivo microdialysis

Rats were anaesthetized with 2.7 ml/kg FFM-mix (2.5 mg/ml fluanisone, 0.079 mg/ml fentanyl citrate and 1.25 mg/ml midazolam,) via a single i.p. injection after s.c. premedication with 0.05 mg/kg atropine sulphate to prevent respiratory arrest and 5 mg/kg carprofen (Rimadyl®) as analgesia. A cannula filled with glycerol/heparin (500 IU/ml) in glycerol was inserted into the femoral artery. The end of the cannula was closed and tunneled to a small opening in the neck, where it was loosely fixed with a ligature. A guide for a CMA10 microdialysis probe (CMA, Sweden) was stereotactically (KOPF Instruments, Tujunga, CA) inserted into the ventral hippocampus (P 0.53, L 3.8, V 2.6 mm) relative to bregma and the dura mater. A heating pad was used to maintain body temperature during surgery and recovery. A CMA microdialysis probe was inserted into the guide at the end of the day and flushed overnight at 2 μl/min with Ringer's solution (147 mM NaCl, 4 mM KCl, 1.5 mM $CaCl_2$ and 0.15 mM HEPES) using a CMA 100 Microdialysis pump. The following day, approximately 14–18 h after probe insertion and recovery from anesthesia, the flow was set back to 0.1 μl/min to approximate maximum recovery, and glycerol/heparin was removed from the arterial cannula to allow administration of tariquidar and clean blood sampling via a syringe attached to the cannula.

Tariquidar (7.5 mg/kg) dissolved in vehicle (propylene glycol:5%dextrose:ethanol 4:5:1) was administered via the arterial cannula, 5 min before an i.m. injection with HI-6 (100 mg/kg; i.m., $n = 5$–6). To determine the uptake of HI6 into the brain hippocampal microdialysates were collected every 20 min for 3 h. Parallel blood samples (150 μl) were drawn from an indwelling catheter in the femoral artery for determination of HI-6 levels.

2.4. Capillary electrophoresis

HI-6 levels were analyzed using capillary electrophoresis in plasma and brain microdialysates using the P/ACE MDQ (Beckman Coulter, Woerden, The Netherlands). In between runs the capillary (ID 75 μm; total length 40 cm, effective length 35 cm) was flushed with NaOH (0.1 M) at 40 psi for dialysate and 50 psi for plasma, HCl (0.05 M) at 20 psi followed by running buffer (200 mM 6-aminohexanoic acid/acetic acid, pH 4.5) at 20 psi. Samples were injected at 1 psi for 10 s (dialysate) or 5 s (plasma). Separation voltage was 15 kV, the absorption wavelength was 292 nm.

In all samples, 2-PAM was used as an internal standard. The dialysates were diluted 5 times in 2-PAM (2 μg/ml in Ringer's solution), yielding a final 2-PAM concentration of 9.2 μM. Plasma samples were diluted 5 times in 20 μg/ml 2-PAM in Ringer's solution containing 60 U/ml heparin, yielding a final 2-PAM concentration of 92 μM. HI-6 concentrations in the samples were calculated using standard curves, and the LOD for HI6 was 3 μM.

2.5. In vivo efficacy studies

Rats were anesthetized with isoflurane (2–3%) and two stainless steel screws (A1.0 and P6.0 mm relative to bregma and 1 mm from the sagittal suture) were placed at the dura mater, fixed to a plug, to serve as EEG electrodes. The electrodes were fixed to the skull with dental cement. Analgesia was provided by s.c. injection of carprofen (Rimadyl®, 5 mg/kg s.c.) during the surgery and 24 h later. The animals were housed in groups until the day of soman challenge.

After 1 week of recovery, the rats were pretreated with HI-6 (125 mg/kg i.p.) 30 min before a s.c. challenge with 200 μg/kg soman. Five minutes before HI-6 injection, rats received an i.v. injection with tariquidar (7.5 mg/kg) or vehicle (propylene glycol/5% sucrose/ethanol 4:5:1) i.v. via the tail vein ($n = 8$/group). At 1 min after soman, animals were treated with atropine sulphate (16 mg/kg i.m.). Cortical EEG was obtained using PhysioTel telemetry system from Data Sciences Inc. (DSI) using the TA11ETA-F40 transmitter body. The transmitter was attached to the plug fixed on the skull of the rat. A receiver board consolidated and stored the signal from the transmitter on an IBM-compatible personal computer via a Data Exchange matrix at 100 Hz sampling frequency. After storage, the data were converted into European Data Format (EDF) and every 10 s power spectra of the EEG were calculated using Fast Fourier Transformation (FFT). The summed power of different frequencies was chosen as representative parameter for the presence of seizures on the EEG signal. Additionally, the start and duration of seizures was manually scored after visual inspection of the EEG signal.

The animals were observed for cholinergic signs of poisoning for 3 h, such as chewing, tremor, salivation and convulsions. Convulsions were defined as involuntary movements of the entire body, while the animal looks dissociated from its environment. After observing either phenomenon, the animal was scored as having the sign, irrespective the duration of the observation. The animals were euthanized by decapitation at 3 h after soman exposure.

2.6. Cholinesterase activity

Brain hemispheres were homogenized (900 rpm, 10% w/v homogenate) in ice-cold TENT buffer, which consisted of 50 mM Tris, 5 mM EDTA, 1 M NaCl and 1% v/v Triton X-100, pH 7.4. The homogenates were centrifuged at 12,000 × *g* in an eppendorf centrifuge at 4 °C and supernatants were immediately frozen in liquid nitrogen and stored at −20 °C until analysis of protein content (Bradford assay) and enzyme activity, within one month. Heparinized blood aliquots were diluted 1:10 (v/v) in 1% saponin and immediately frozen in liquid nitrogen.

Samples were analyzed for ChE activity using a modification of the method by Ellman et al. (1961). Shortly, after appropriate dilution, 10 μl blood or homogenate samples were incubated with 0.8 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) (Sigma Aldrich B.V.) and 0.8 mM acetylthiocholine iodide. Absorbance at 415 nm was measured directly after the addition of the acetylthiocholine substrate and 40 min later. During this time, the assay was linear. The increase in absorbance per μl sample (blood) or mg protein (homogenates) per min at ambient temperature served as measurement for ChE activity.

2.7. Data presentation and statistical analysis

All data are presented as means ± SEM. Statistical analysis was performed by Student's unpaired *t*-test and Two-Way ANOVA where appropriate. Results were considered significant for $p < 0.05$.

3. Results and discussion

3.1. In vivo microdialysis

The primary *in vivo* experiments using microdialysis in the hippocampus of freely moving rats showed that pretreatment of rats with tariquidar 15 min before an i.m. injection of HI-6 did not influence the HI-6 levels in plasma (Fig. 1). However, in the hippocampus, a nearly 2-fold increase of HI-6 levels was observed, and the overall HI-6 levels in the brain of tariquidar pretreated animals appeared to be higher than in controls ($p < 0.05$, Fig. 1b). Whereas the $AUC_{0-1\,h}$ of HI-6 in the brain had significantly increased approximately 2-fold in the tariquidar pretreated group (Fig. 1c), the $AUC_{0-3\,h}$ levels were not significantly different between groups. The latter observation points to a more pronounced effect of tariquidar on HI-6 levels shortly after oxime administration.

Similar levels of increase of brain drug levels after Pgp inhibition by tariquidar were found for the small molecules phenytoin and paclitaxel (Hubensack et al., 2008; van Vliet et al., 2006). Pgp blocking by tariquidar is highly effective, and directly related to plasma